THE EFFECT OF HYDROCORTISONE ON THE PRODUCTION OF HERPES SIMPLEX VIRUS IN TISSUE CULTURE*

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Cortisone and hydrocortisone have an adverse effect upon some viral infections of man (1–6) and upon a number of experimental viral infections in laboratory animals (7–21). Treatment of laboratory animals with these steroids often results in more severe symptoms and at times in a higher mortality rate and earlier death. In some instances in both animals and man an infection which is ordinarily mild or almost undetectable is transformed into a serious or fatal disorder (1–6, 12, 13, 15, 16, 21).

Recurrent herpes simplex infections of the cornea of man are aggravated and sometimes precipitated by the topical use of cortisone or hydrocortisone (4–6). Topical treatment with these steroids results in a more severe, a deeper and a more prolonged infection which occasionally leads to perforation of the cornea and panophthalmitis. Experimental herpetic infection of the rabbit cornea is more severe in animals treated locally or systemically with cortisone or hydrocortisone (7–10). The herpetic keratoconjunctivitis of the untreated animals begins to resolve in 7 to 10 days and gradually heals. The keratoconjunctivitis of the cortisone or hydrocortisone-treated rabbit fails to improve at the 7 to 10 day period. Instead, the infection becomes gradually more extensive and severe ophthalmitis develops.

HeLa cell tissue cultures infected with herpes simplex virus were chosen as a model to study the effect of hydrocortisone upon the course of the herpetic infection and the amount of herpes simplex virus produced. Under the conditions of this study hydrocortisone had no influence on the amount of virus produced. There was, however, considerable effect of the hormone on the microscopic appearance of the culture.

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MATERIALS AND METHODS

A. Cells—HeLa cells, which had been passed serially in our laboratory, were used exclusively. Cells were grown in 16 × 125 mm. screwcapped tubes in stationary racks at 35°C (22).

B. Growth Medium—Two types of growth media were employed: (1) Eagle's basal medium containing 40% human serum was used in Experiments 1–6 and (2) One-tenth per cent yeast extract medium (23) containing 20% human serum was used in Experiments 7 and 8.

C. Maintenance Medium—A single maintenance medium, designated HSMS, was employed. It consisted of 10% horse serum, salts, vitamins, amino acids, phenol red, penicillin and streptomycin (22).

D. Virus—Two strains (22) of herpes simplex virus were employed: (1) HF which was obtained from the American Type Culture Collection was used in Experiments 1 and 2 and (2) ST which was isolated from a recurrent herpes lesion of the thigh of a 23 year old woman was used in Experiments 3 to 8. Stock virus was prepared by serial passage in HeLa cultures.

E. Titration of Virus—Virus titrations were performed by a plaque count method which has been described (22). The method consisted essentially of the addition of aliquots of serial dilutions of the virus suspension to groups of HeLa cultures. The number of plaques produced at the end of 48 hours at the lowest dilution where plaque counts were possible was then used to calculate the virus titer by the formula n equals Y/VX where n equals the number of plaque forming units of virus per ml., Y equals the number of plaques counted, V equals the volume of the suspension in ml. and X equals the dilution at which the plaque counts were done. In Experiments 1 through 7 no attempt was made to separate virus associated with the cells from virus in cell-free medium, i.e., titrations were done on uncentrifuged cell-containing medium which was frozen and thawed four times. In Experiment 8 all samples to be titrated for virus were first centrifuged at 3,000 r.p.m. for ten minutes. The supernatant fluid and sedimented portions of these samples were stored separately and titrated separately for virus (cell-free medium and cell-containing medium of Experiment 8).

F. Storage of Virus—Virus suspensions were

stored in the CO_2 box (−45°C) for short periods of time (two days to two weeks) before titration of virus content. Titrations of steroid-treated and corresponding non-treated cultures were always done simultaneously to minimize experimental error.

G. Hydrocortisone—Three hydrocortisone preparations were used: (1) Upjohn Cortef Hemisuccinate Sodium, Lot No. GP-518 was used in Experiments 1, 2, 3, 4, and 5. (2) Merck and Co. Hydrocortisone Free Alcohol No. 54R4095 was used in Experiment 6. (3) Upjohn Solu-Cortef buffered with sodium biphosphate and sodium phosphate was used in Experiment 7 and 8. Hydrocortisone (1) and hydrocortisone (2) were available in powder form. Hydrocortisone (3) was dissolved in the diluent provided with the ordinary commercial container. Stock solutions of concentrated hydrocortisone were prepared with growth or maintenance medium. These were later mixed with the culture media to obtain concentrations of 1, 5 or 10 micrograms of hydrocortisone per ml. This concentration of hydrocortisone was chosen because it was thought it would correspond to expected serum levels in patients treated with hydrocortisone. In Experiment 5 hydrocortisone was added to the growth medium of some of the cultures for three days before inoculation of the virus and the addition of maintenance medium. In all other experiments hydrocortisone was present only in the maintenance medium.

H. Procedure for Infection of Cultures—Cultures were nourished with growth medium until solid monolayer sheets of cells were seen on the glass walls of the culture tubes. The growth medium was then decanted and the cultures were washed

EXPERIMENT NO. 1 - **Plaque Counts and Virus Titers of HF Herpes-Infected HeLa Cultures Nourished With Maintenance Medium Containing Varying Amounts of Hydrocortisone**

Hydrocortisone per ml. of medium	Plaque counts 48 hours after inoculation of virus		Virus titers of pooled cells and medium four days after inoculation of virus
	Counts	Average	
None	14, 17, 6, 11, 13, 7	11	$10^{3.48}$
1 microgram	7, 5, 7, 16, 11, 18	11	$10^{3.74}$
5 micrograms	25, 25, 15, 10, 6	16	$10^{3.82}$
10 micrograms	4, 6, 6, 8, 19	9	$10^{3.81}$

EXPERIMENT NO. 2 - Plaque Counts and Virus Titers of HF Herpes-Infected HeLa Cultures Nourished With Maintenance Medium Containing Varying Amounts of Hydrocortisone

Hydrocortisone per ml. of medium	Plaque counts 48 hours after inoculation of virus		Virus titers of		
	Counts	Average	Decanted medium at 2 days	4 days	Pooled cells and medium at 6 days
None	32, 35, 19, 32, 16	27	$10^{0.0}$	$10^{3.62}$	$10^{5.16}$
None	15, 29, 41, 44, 31	32	$10^{0.48}$	$10^{3.76}$	$10^{5.28}$
1 microgram	34, 36, 36, 33, 37	35	$10^{0.0}$	$10^{3.69}$	$10^{5.87}$
5 micrograms	26, 35, 34, 12, 46	31	$10^{0.48}$	$10^{3.96}$	$10^{5.98}$
10 micrograms	28, 32, 43, 15, 33	30	$10^{0.48}$	$10^{3.95}$	$10^{6.02}$

EXPERIMENT NO. 3 - Plaque Counts and Virus Titers of ST Herpes-Infected HeLa Cultures Nourished With Maintenance Medium Containing Varying Amounts of Hydrocortisone

Hydrocortisone per ml. of medium	Plaque counts 48 hours after inoculation of virus		Virus titer of	
	Counts	Average	Decanted medium at 2 days	Pooled cells and medium at 4 days
None	58, 70, 42, 73, 51	59	$10^{3.47}$	$10^{5.94}$
None	66, 54, 41, 47, 52	52	$10^{3.75}$	$10^{5.93}$
1 microgram	62, 67, 68, 50, 40	57	$10^{3.51}$	$10^{6.01}$
5 micrograms	67, 40, 55, 33, 47	49	$10^{3.42}$	$10^{5.73}$
10 micrograms	52, 56, 52, 52, 46	52	$10^{3.42}$	$10^{5.71}$

EXPERIMENT NO. 4 - Virus Titers of ST Herpes-Infected HeLa Cultures Nourished With Maintenance Medium Containing Varying Amounts of Hydrocortisone. Hydrocortisone Added Two Days Before Inoculation of 15 pfu of Virus Per Culture

Hydrocortisone per ml. of medium	Virus titer of pooled cells and medium 5 days after addition of steroid and 3 days after inoculation of virus
None	$10^{3.89}$
None	$10^{3.87}$
1 microgram	$10^{4.28}$
5 micrograms	$10^{4.0}$
10 micrograms	$10^{4.25}$

EXPERIMENT NO. 5 - Plaque Counts and Virus Titers of ST Herpes-Infected HeLa Cultures Nourished With Growth and Maintenance Medium Containing Varying Amounts of Hydrocortisone

Hydrocortisone per ml. of growth medium for 3 days prior to virus inoculation	Hydrocortisone per ml. of maintenance medium added at virus inoculation	Plaque counts 48 hours after inoculation of virus		Virus titers of	
		Counts	Average	Decanted medium at 3 days	Pooled cells and medium at 5 days
None	None	59, 65, 54	59	$10^{4.23}$	$10^{5.89}$
	1 microgram	57, 65, 64, 66	63	$10^{4.20}$	$10^{5.95}$
	10 micrograms	69, 67, 52, 53	60	$10^{4.45}$	$10^{6.25}$
1 microgram	None	35, 41, 35, 40	38	$10^{3.74}$	$10^{5.97}$
	1 microgram	72, 54, 43, 36	51	$10^{4.04}$	$10^{6.20}$
10 micrograms	None	36, 35, 35, 38	38	$10^{4.71}$	$10^{6.30}$
	10 micrograms	56, 51, 36, 34	44	$10^{4.11}$	$10^{6.18}$

EXPERIMENT NO. 6 - Virus Titers of ST Herpes-Infected HeLa Cultures Nourished With Maintenance Medium Containing Varying Amounts of Hydrocortisone

Hydrocortisone per ml. of medium	Virus titer of pooled cells and medium 2 days after inoculation with 10^4 pfu of virus per culture
None	$10^{5.86}$
None	$10^{5.89}$
1 microgram	$10^{6.11}$
5 micrograms	$10^{5.90}$
10 micrograms	$10^{5.91}$

EXPERIMENT NO. 7 - Plaque Counts and Virus Titers of ST Herpes-Infected HeLa Cultures Nourished With Maintenance Medium Containing Varying Amounts of Hydrocortisone

Hydrocortisone per ml. of medium	Plaque counts 48 hours after inoculation of virus		Virus titers of cells and medium from pooled paired cultures at 2 days					
	Counts	Average						
None	X, 141, 115, 120, 131, 94, 111, 85, 93, 63, 83, 91	103	$10^{5.48}$	$10^{5.75}$	$10^{5.72}$	$10^{5.69}$	$10^{5.69}$	$10^{5.40}$
1 microgram	99, 94, 97, 96, 78, 87, 88, 70, 91, 71, 79, 62	85	$10^{5.08}$	$10^{4.82}$	$10^{4.45}$	$10^{4.42}$	$10^{4.30}$	$10^{4.55}$
5 micrograms	103, 101, 79, 76, 60, 83, 103, 100, 75, 98, 95, 86	89	$10^{4.72}$	$10^{5.11}$	$10^{4.36}$	$10^{4.73}$	$10^{4.66}$	$10^{4.67}$
10 micrograms	100, 104, 97, 76, 109, 76, 58, 66, 58, 63, 46, 54	76	$10^{4.64}$	$10^{4.0}$	$10^{4.36}$	$10^{4.86}$	$10^{4.83}$	$10^{4.68}$

X = unsatisfactory plaque count

EXPERIMENT NO. 8 - Plaque Counts and Virus Titers of ST Herpes-Infected HeLa Cultures Nourished With Maintenance Medium Containing Varying Amounts of Hydrocortisone

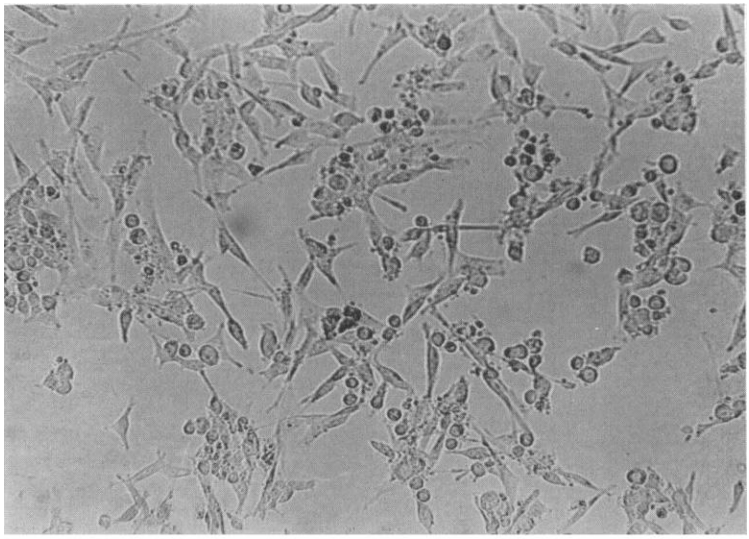
Hydrocortisone per ml. of medium	Plaque counts 48 hours after inoculation of virus		Virus titers of									
			Cell-free medium at					Cell-containing medium at				
	Counts	Average	1 day	2 days	3 days	4 days	5 days	1 day	2 days	3 days	4 days	5 days
None	87, 88, 105, X, 85, 109, 74, 73	89	$10^{1.0}$	$10^{2.30}$	$10^{4.62}$	$10^{5.60}$	$10^{6.21}$	$10^{2.63}$	$10^{5.19}$	$10^{6.27}$	$10^{7.34}$	$10^{6.60}$
1 microgram	76, 88, 105, 69, 96, 84, 97, 87	88	$10^{0.69}$	$10^{3.08}$	$10^{4.28}$	$10^{5.61}$	no sample	$10^{2.80}$	$10^{5.16}$	$10^{5.76}$	$10^{7.14}$	$10^{6.68}$
5 micrograms	73, 91, 77, 86, 81, 67, 89, 97	83	$10^{0.69}$	$10^{2.69}$	$10^{4.07}$	$10^{5.69}$	$10^{5.88}$	$10^{2.36}$	$10^{4.55}$	$10^{5.86}$	$10^{7.11}$	$10^{6.36}$
10 micrograms	96, 75, 71, 78, 95, 80, 75, X	81	$10^{0.0}$	$10^{2.72}$	$10^{4.16}$	$10^{5.40}$	no sample	$10^{2.43}$	$10^{4.40}$	$10^{6.03}$	$10^{6.89}$	$10^{6.15}$

X = unsatisfactory plaque count

two times with 2 ml. of Hank's balanced salt solution. The virus suspension was then added to the cultures in one-tenth ml. amounts. One to two hours was allowed for attachment of the virus to the cells. The maintenance medium (with or without hydrocortisone) was then added to the cultures and they were incubated in slant racks at 35°C for varying periods of time (see Experiments 1–8).

RESULTS

A total of eight experiments was performed and the results are presented in table form. Hydrocortisone in the maintenance medium did not consistently influence the amount of herpes virus produced by HeLa cultures (Experiments 1–8). In some instances it appeared



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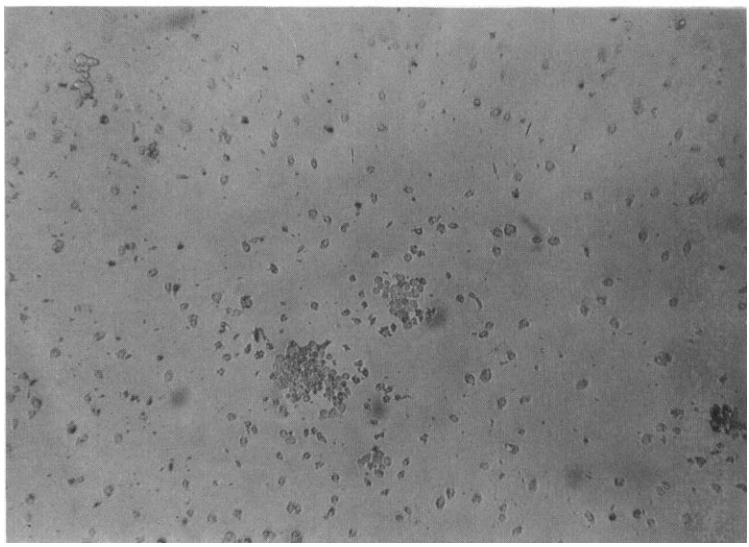

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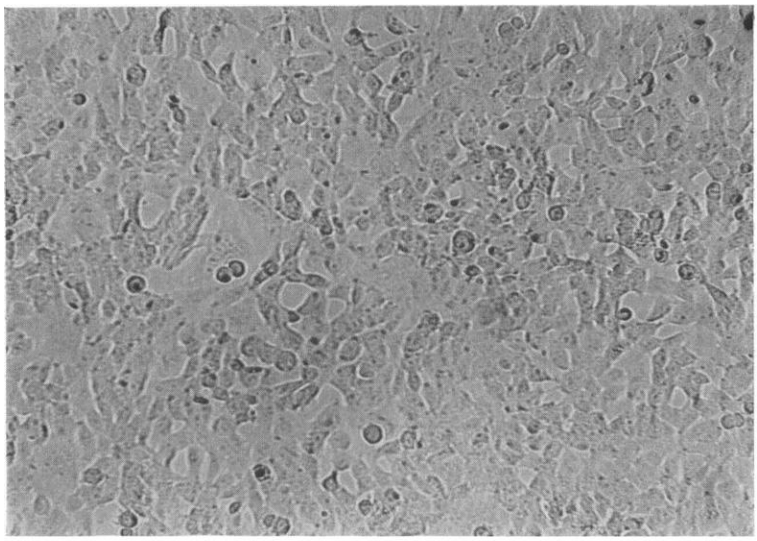
FIG. 1. Control HeLa cell culture (Experiment No. 2) after six days on maintenance medium without hydrocortisone. Moderately severe degenerative changes. × 100

FIG. 2. Control HeLa cell culture (Experiment No. 4) after five days on maintenance medium without hydrocortisone. Severe degenerative changes present. × 50



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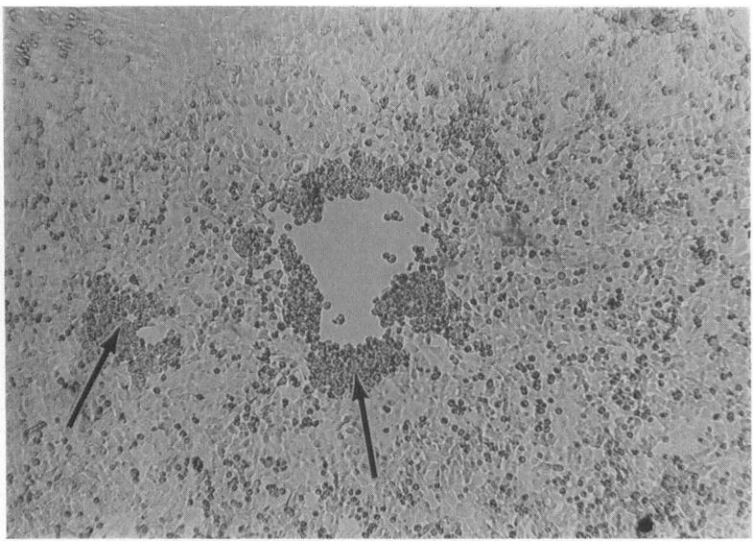

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FIG. 3. HeLa cell cultures (Experiment No. 2) after six days on maintenance medium containing one gamma hydrocortisone per ml. "Preservative" effect of steroid is evident. × 100

FIG. 4. HeLa cell culture (Experiment No. 4) after five days on maintenance medium containing one gamma of hydrocortisone per ml. ST herpes virus was added on the third day of maintenance. Arrows point to herpes-infected cells. "Preservative" effect on culture is evident. × 50

that slightly more virus was produced and in other instances slightly less than in the control cultures. The differences, however, were not consistent and they were so small that they should be regarded as within the experimental error of the methods involved. (A significant difference would require consistent variations of one log or more in the virus titers).

Plaque counts were not significantly different whether hydrocortisone was present in the maintenance medium or not. However, plaque counts were often easier to make in the presence of hydrocortisone since, as will be mentioned below, the cell sheet was more solid and showed less degenerative change.

Hydrocortisone in the maintenance medium had a pronounced effect upon the microscopic appearance of the HeLa cultures. This effect

was primarily to delay and decrease "non-specific" degenerative changes in the cultures under maintenance conditions. Under "good" conditions degenerative changes of mild degree developed in the cultures in 3 to 7 days and these became rather severe thereafter (Fig. 1). Under "poor" conditions (which include "toxic" glassware, "poor" lots of serum, etc.) cells showed degenerative changes in a day or two and these became severe in three or four days (Fig. 2). The degeneration was manifested by the cells becoming round, spindle-shaped or granular and of their falling off the glass as individual cells, small clumps or large patches of cells. Hydrocortisone in the medium delayed the appearance of the degenerative changes by one to three days or more and when they developed they were much less pronounced (Fig. 3 and Fig. 4). At times cultures on maintenance with hydrocortisone looked almost as good after a week or two or even for as long as 25 days as they did when they were placed on maintenance medium. Control cultures at a week or two usually showed severe degenerative changes. At times 90–99% of the cells would have fallen off the glass wall of the culture tube in the control whereas the hydrocortisone treated tube still had 90% or more cells still on the glass (Fig. 2 and Fig. 4). The hydrocortisone-treated cultures at most times were more acid than the controls judging from the color of the phenol red indicator. Part of the increased acidity may have been due to a greater number of cells in the hydrocortisone cultures (cells had dropped off the controls and had been discarded with changes of media) but at least some of the acidity appeared to be a hydrocortisone effect on the cells. Cultures kept in hydrocortisone-containing maintenance medium for as long as 25 days could still develop herpetic cytopathogenesis when infected with herpes virus.

DISCUSSION

Although hydrocortisone in small amounts in the maintenance medium did not influence the amount of herpes virus produced, it did exert a preservative effect upon the HeLa cell cultures. Since the preservative effect did not interfere with multiplication of the virus or with plaque formation, the possibility arises that some tissue culture procedures involving virus studies may be benefited by the addition of hydrocortisone to the culture medium

The mechanism whereby cortisone or hydrocortisone enhances herpes simplex infections of the eye (or for that matter any other viral infection) is incompletely understood. The question arises whether there is more herpes virus produced under the influence of these steroids. Jawetz *et al* (10) found no evidence that treatment with hydrocortisone increased virus production in rabbits infected with herpes virus by the corneal route. There was no evidence that hydrocortisone caused increased virus production in the herpes virus-HeLa cell model described herein. This suggests that some mechanism or mechanisms other than increased production of virus may be operating in steroid aggravation of the herpetic infection.

Theoretically cortisone or hydrocortisone might enhance viral infection in a number of ways (16) including: (1) Direct effect on the virus. (2) Increased virus production. (3) Inhibition of antibody formation. (4) Inhibition of inflammatory and reparative reactions and (5) others. In regard to these points the following comments can be made: Mixing extracellular virus and hydrocortisone has had no effect on the virus (20). Cortisone or hydrocortisone aggravation of some viral infections is associated with increased production of virus but in others it is not (10, 11, 12, 14, 16, 17). It would be possible for these steroids to enhance viral infection by interfering with antibody production but often the adverse effect of the steroid is found before antibody production would be expected to play a significant role or in systems such as embryonated eggs where antibody production would not be expected to occur at all (11, 17, 18, 20). Cortisone or hydrocortisone may enhance viral infections by inhibiting inflammation and repair. Under various conditions cortisone or hydrocortisone has been shown to have the following effects on cells, tissues or organisms: (1) The growth of chick embryos is decreased (11, 24). (2) Granulation tissue in wounds is inhibited (25, 26). (3) The outgrowth of fibroblasts from tissue explants is decreased (27–29). (4) Growth and division of cells in tissue culture is decreased (30). (5) The inhibiting effect on growth and migration is less for epithelial cells than for connective tissue cells (26, 29). (6) Respiration and glycolysis of cells in tissue cultures is changed (30, 31). (7) Morphologic changes of cells in tissue culture occur (32).

SUMMARY

1. The amount of herpes simplex virus produced in HeLa cultures under maintenance conditions was not influenced by the presence of hydrocortisone in the medium in amounts of 1, 5 and 10 micrograms per ml.

2. Hydrocortisone in the maintenance medium had a pronounced effect in delaying and decreasing "non-specific" degenerative changes of HeLa cell cultures under the conditions of this study.

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